Exhibit 2

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Preferential Activation of Th2 Cells in Chronic Graft-versus-Host Reaction[1]

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ABSTRACT. The injection of DBA/2 parental lymphocytes into adult, immunologically intact (C57BL/6 × DBA/2) F_1 hybrid mice results in a chronic graft-vs-host reaction (GVHR) characterized by a deficiency in $CD4^+$ T cell functions and a B cell activation leading to autoantibody production. The discovery that distinct subpopulations of Th cells may regulate the effector immune functions led us to investigate whether the chronic GVHR differentially affects Th subsets. Data are presented indicating that mice undergoing a GVHR spontaneously produced lymphokines of Th2 origin. IL-4 and IL-10 mRNA were detected in the spleens of GVH mice, and IL-4 was shown to be responsible for the increased expression of class II Ag on B cells. Moreover, upon polyclonal activation in vitro, GVH T cells exhibited defective IL-2 and IFN-γ production but elevated IL-4 production. We conclude that the chronic GVHR is characterized by a selective deficiency in cells secreting IL-2 and IFN-γ and a hyperactivation of Th2 cells. The simultaneous production of IL-4 and IL-10 might explain the association between B cell hyperactivity and impairment of Th1-like activities in various models that associate autoimmunity and immunosuppression, such as GVHR and HIV infection. *Journal of Immunology,* 1993, 150: 361.

A single inoculation of parental cells into an unirradiated, immunocompetent F_1 recipient induces profound defects in the host immune response. Depending on both MHC disparity and non-MHC-linked loci, GVHR[3] can be found in acute or chronic form. The acute GVHR is characterized by complete abrogation of immune reactivity and repopulation of host spleen by donor T cells, whereas the chronic GVHR is characterized by a less severe immunodeficiency (1, 2).

Indeed, studies investigating in vitro responses of chronic GVHR have demonstrated a selective loss of $CD4^+$ Th functions and IL-2 production (3, 4). In these animals, however, $CD8^+$ functions (cytolytic and helper) remain intact, as shown by the ability of mice undergoing a chronic GVH to mount allospecific, CD4-independent, class I-restricted CTL responses (3, 4).

This selective deficiency of $CD4^+$ cells is associated with hyperactivation of B cell functions leading to production of autoantibodies, antibody-mediated glomerulonephritis and hypergammaglobulinemia (1). In particular, a major increase in IgE serum levels was observed early after chronic GVH induction. In vivo administration of an anti-murine IL-4 mAb completely prevented the hyper IgE

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[3] Abbreviations used in this paper: GVHR, graft-vs-host reaction; PCR, polymerase chain reaction; sIgM, surface IgM.

production, showing that IL-4 is one of the mediators of B cell activation in this system (5).

Thus, the chronic GVHR appears to affect differently the B and T cell functions in vivo. Based on recent studies showing that Th1 and Th2 cells have opposite effects on immune responses, we hypothesized that both subsets could play a role in the dysfunction observed in mice undergoing a chronic GVHR. Therefore, we analyzed the lymphokine profile (6, 7) of cells isolated from animals undergoing a chronic GVHR in order to better define the cellular interactions leading to the selective T cell deficiency and to the B cell hyperactivation.

Material and Methods

Mice

Female DBA/2 mice (H-2^d) and (C57BL/6 × DBA/2) F_1 mice (abbreviated B6D2, H-$2^{d/b}$) were purchased from Charles River Wiga (Sulzfeld, Germany).

Induction of GVHR

Cell suspensions were prepared in RPMI from the spleens of DBA/2 mice and 8×10^7 cells were injected via the tail vein into normal (unirradiated) F_1 hybrid mice. Some mice received two weekly injections of donor cells, as indicated in the legend to Table I. Control mice were either untreated or injected with 8×10^7 syngeneic F_1 spleen cells.

mAb

Culture supernatants from the following lines were used as sources of antibodies as previously described (8): anti-CD3, 145-2C11; anti-Thy-1.2, HO.13.4; anti-IL-4, 11B11; anti-IL-10, SXC1 (a gift from Dr. T. Mosmann, University of Alberta, Alberta, Canada); and anti-K^b, 28.8.6 or 28.13.3 (kindly provided by Dr. D. Sachs, Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD).

Immunofluorescence

Single and two-color analyses were performed as previously described (9).

Isolation of donor and host T cells from GVH mice

Splenic cells from control and GVH mice were stained with rabbit anti-mouse Ig antibodies coupled to biotin and passaged over a Magnetic Cell Sorter column (Miltenyi Biotec, Bergisch-Gladbach, Germany), according to the manufacturer's recommendations. The eluted cells (at least 90% T cells) were then stained with anti-K^b mAb (28.8.6) followed by fluoresceinated anti-IgG2a antibodies. Donor (K^b negative) and host (K^b-positive) cells were separated by sorting (FACS Star, Becton Dickinson, Mountain View, CA). Alternatively, donor T cells were obtained by lysis of host cells with anti-K^b mAb (28.13.3) and C (young rabbit serum selected in our laboratory). Enriched donor and host populations contain less than 10% contaminant cells (data not shown).

In vitro experiments

A total of 10^7 unseparated spleen cells were incubated in 24-well plates in the presence or absence of anti-CD3 mAb (145.2C11) at a final concentration of 1.5 μg/ml. Supernatants from anti-CD3-stimulated cultures were assayed for IL-2 after 24 h of incubation and for IL-5 and IFN-γ after 72 h of incubation.

Lymphokines assays

IL-2 and IL-4 productions were measured as previously described (8). IFN-γ was quantitated by two-site ELISA using mAb F_1 and Db-1, kindly provided by Dr. Billiau (K.U.L., Leuven, Belgium), and P. H. Van Der Meide (TNO Health Research, Rijswijk, The Netherlands), respectively.

PCR analysis of cytokines genes expression

RNA was extracted from mouse spleen cells using the guanidine thiocyanate method (10). After preparation of cDNA, PCR was performed essentially as previously described (11). Briefly, 5 μg of total RNA was incubated 10 min at 65°C with 10 μg oligo$(dT)_{15}$ and further incubated for 60 min at 37°C with 120 U RNAsin (Promega Biotec, Madison, WI), 1 mM dNTP, 1000 U Moloney murine leukemia virus reverse transcriptase, 100 μg/ml acetylated BSA, and reverse transcriptase buffer (75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol 50 mM Tris HCl, pH 8.3) in final volume of 100 μl. PCR was performed using aliquots of the resulting cDNA (equivalent to 100 to 500 ng of total RNA). To this was added 200 μM dNTP, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl (pH 8.3), 1 mg/ml gelatine, 2.5 U Taq DNA polymerase (Perkin-Elmer/Cetus, Norwalk, CT) and 1 μg of each sense/antisense primer in a total volume of 25 μl. Reactions were incubated in a Perkin-Elmer/Cetus DNA thermal cycler for 25 cycles (denaturation: 1 min, 93°C; annealing: 2 min, 55°C; extension: 3 min, 72°C). Primers used were as follows: IL-2 sense primer 5′-TGATGGACCTACAGGAGCTCCTGAG-3′ and antisense 5′-GAGTCAAATCCAGAACATGCCGCAG-3′ (amplified fragment of 168 bp). IL-4 sense primer 5′-AACACCACAGAGAGTGAGCTCGTCT-3′ and antisense 5′-TGGACTCATTCATGGTGCAGCTTAT-3′ (amplified fragment of 178 bp). IFN-γ sense primer 5′-AGCGGCTGACTGAACTCAGATTGTAG-3′ and antisense 5′-GTCACAGTTTTCAGCTGTATAGGG-3′ (amplified fragment of 244 bp). IL-10 sense primers (5′-TCAAACAAAGGACCAGCTGGAC-

Table 1
Class II expression on B cells during stimulatory GVHR[a]

sIgM⁺ Cells from	Weeks after First Injection of DBA/2 Spleen Cells		
	2	6	12
Spleen	340 ± 4[a,b] (100 ± 7)	408 ± 48[b] (100 ± 6)	217 ± 57[b] (100 ± 5)
Lymph node	220 ± 31[b] (100 ± 16)	323 ± 59[b] (100 ± 16)	231 ± 52[b] (100 ± 7)
Peripheral blood	293 ± 56[b] (100 ± 7)	308 ± 27[b] (100 ± 4)	220 ± 97[b] (100 ± 4)

[a] Mean fluorescence intensity of class II expression on sIgM⁺ cells, expressed as percentage of the values observed in control mice (mean ± SD). Five to seven mice were tested in each group. Stimulatory GVH was induced in (C57BL/6 x DBA/2) F_1 mice by two i.v. injections of 10^8 DBA/2 spleen cells at week 0 and week 1.
[b] $p < 0.01$ as compared with control uninjected mice.

AACATACTGC-3′ and antisense 5′-CTGTCTAGGTC-CTGGAGTCCAGCAGACTCAA-3′ (amplified fragment of 421 bp). HPRT sense primer 5′-GTTGGATACAGGC-CAGACTTTGTTG-3′ and antisense 5′-GATTCAACTT-GCGCTCATCTTAGGC-3′ (amplified fragment 163 bp).

Results

Cells from GVH mice express high levels of class II Ag

The expression of class II Ag on B cells bearing sIgM⁺ was evaluated by double color staining. Results presented in Table I indicate that B cells from GVH mice displayed a two- to fourfold increase in the expression of class II Ag. This phenomenon was maximal 6 wk after GVH induction and was observed in the spleen, the lymph nodes, and the peripheral blood (Table I).

Anti-IL-4 inhibits overexpression of class II

Because we have previously shown that IL-4 was responsible for the hyper-IgE secretion (5), we investigated the role of IL-4 in the increase of class II expression in vitro. As shown in Figure 1*a*, spleen cells isolated from GVH animals and cultured for 24 h express high levels of class II Ag. The hyper-class II expression is prevented by addition of anti-IL-4 mAb in the media, showing that cells from GVH mice spontaneously produce high levels of IL-4 that is responsible for the increased class II expression.

Donor T cells are the major source of IL-4 in vitro

Chimeric T cells can be detected in chronic GVH. In this system, it has been shown that donor T cells (of DBA/2 origin) were present in the spleen of B6D2 GVH mice and were almost exclusively of the CD4⁺ phenotype (4). In order to identify the cells producing IL-4 in this system, donor (DBA/2 origin) and recipient (B6D2 F_1 origin) T

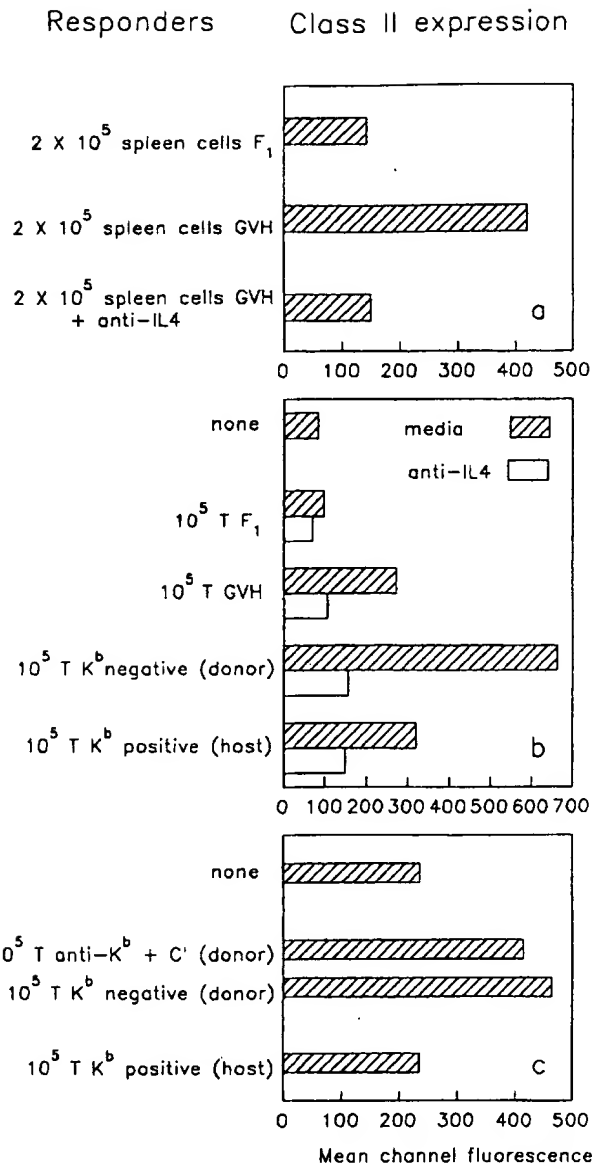

FIGURE 1. Donor T cells are the major source of IL-4 in vitro. Spleen cells from F_1 or GVH mice are cultured for 24 h in complete medium with or without anti-IL-4 mAb (11B11) and the expression of class II determinants is analyzed by flow cytometry (*a*). Thy-1 depleted DBA/2 spleen cells were cultured with whole T cells from control and GVH mice, or with donor and host T cells isolated from the spleens of GVH mice (see *Materials and Methods*). Production of IL-4 was evaluated by the intensity of class II expression after 18 h of culture with or without anti-IL-4 mAb (*b*). The same experiment was performed using donor T cells obtained either by lysis with anti-K^b + C, or negatively sorted by FACS (see *Materials and Methods*) (*c*).

cells were purified and cultured in the presence of indicator B cells as previously described (8). The experiments shown in Figure 1 *b* and *c* indicate that most of the IL-4 is produced by donor cells. Indeed, donor cells, enriched by negative selection (anti-K^b + C) or purified by cell sorting